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Sexual dimorphism in the genus *Leucadendron*: Morphology and plant hydraulics

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Summary

- The genus, *Leucadendron,* of the Cape Proteaceae family, is made up of over 70 dioecious species that vary in their degree of sexual dimorphism. Males are generally more highly ramified (branched) with smaller leaves compared to corresponding females.
- It has been hypothesised that sexual dimorphism in *Leucadendrons* is linked to serotiny (a fire-adapted reproductive strategy), where highly serotinous females may incur extra resource costs in order to keep their transpiring cones alive between fires. This hypothesis predicts that the female morphology might be associated with more efficient resource acquisition compared to males in order to support their greater resource requirements. Another hypothesis suggests that selection for greater floral display in males has lead to a higher degree of ramification as male cones are borne terminally on branches. This highly branched morphology may be associated with subsequent physiological costs.
- The idea that different male and female morphologies might be associated with different physiological costs or benefits was tested in this experiment with a focus on plant hydraulics. Hydraulic supply is known to affect photosynthetic capacity and maximum assimilation rate.
- Using a specially designed vacuum chamber, leaf-specific and xylem-specific hydraulic conductance was measured in males and females of the highly dimorphic *Leucadendron rubrum* and non/marginally dimorphic *Leucadendron daphnoides*. Using microscopic imagery, xylem anatomy was analysed in an attempt to explain the hydraulic conductance results.
- Male and female *L. rubrum* individuals were highly dimorphic and differed significantly in their leaf size and degree of ramification. Males had smaller leaves and were more highly ramified (branched) than females. *L. daphnoides* males and females were only marginally dimorphic and had similar leaf size and degree of ramification.
- The highly dimorphic morphologies of males and females in *L. rubrum* were associated with significant hydraulic conductance differences. Females were found to have significantly higher hydraulic conductance (leaf-specific & xylem specific) than males. The morphologically similar *L. daphnoides* males and females had similar hydraulic conductance values.
- Higher conductivity in *L. rubrum* females was associated with larger average vessel size. Average vessel size in *L. daphnoides* males and females was similar.
- Hydraulic conductance differences between males and females appeared to be associated with a high degree of sexual dimorphism. This empirical evidence suggests that in highly dimorphic species such as *L. rubrum*, highly ramified males may be physiologically disadvantaged compared to females. This can be used to support a number of current hypotheses that attempt to explain the reasons for sexual dimorphism.

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Introduction

The majority of angiosperms are monoecious, meaning that a single plant holds both male and female reproductive organs. More rarely, species exist with separate male and female plants. This condition is known as dioecy and it occurs in close to half of all angiosperm families (Heilbuth 2000). Among dioecious plants, males and females often differ to varying degrees in terms of their morphology which may affect physiology and life-history traits (Barret and Hough 2013). This phenomenon is known as sexual dimorphism.

Darwin (1871) proposed that in animals, the evolution of sexual dimorphism was the result of variation in mating success among individuals. This was thought to occur in two ways. The first is intrasexual competition among individuals of one sex (usually males) for mates of the other sex. The second is intersexual mate choice, where individuals of one sex (usually females) chose a mate from the other sex based on favourable traits. This concept was extended by Bateman's principle, which states that the reproductive success of males is usually limited by the availability of mating partners. Female reproductive success, on the other hand, is more likely to be limited by resources (Bateman 1948). Darwin's (1871) work did not address the possibility of sexual selection occurring in plants, however it is now generally accepted that Batemans's principals can be used to describe sexual dimorphism in many angiosperms (Barret and Hough 2013). The application of Bateman's principle to angiosperms is associated with some controversy however.

The genus *Leucadendron,* of the Proteaceae family, is made up of over seventy dioecious species that vary in their degree of sexual dimorphism (Williams 1972). Males are generally more highly ramified (branched) with thinner branches and smaller leaves compared to corresponding females (Rebelo 2001). It is not yet known to what extent sexual dimorphism affects physiology, life-history and resource expenditure in male and female *Leucadendrons*. We might expect that vast differences in these areas should lead to the divergence in the geographical distribution of males and females, yet they appear to co-occur closely in the landscape. Geographical variation in sex ratios and the occupation of different niches between sexes in heterogenous environments have been recorded in sexually dimorphic angiosperm populations (Barret and Hough 2013). The selective pressures driving the morphological divergence of males and females are also not fully understood, although a

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number of hypotheses exist that attempt to explain the high occurrence of sexual dimorphism in the *Leucadendron* genus.

In the *Leucadendron* genus, species exhibit a varying degree of serotiny, which is a fire-adapted, reproductive strategy whereby females store seeds in closed cones and release them only after fire. While some species support their closed cones for a limited period of time only, highly serotinous species hold their seeds in closed cones for a number of years between fires. Seeing that these seed-baring cones must transpire to be kept alive (Midgley 2000), it has been hypothesised that females with a high degree of serotiny incur extra reproductive costs. Harris and Pannell 2010 found that the degree of sexual dimorphism in *Leucadendrons* strongly correlates to the degree of serotiny and that more serotinous species are the most dimorphic. They further suggested that highly serotinous females may have evolved a different, more efficient morphology to males, in order to support the extra reproductive costs associated with keeping cones alive (Harris and Pannell 2010).

Other studies suggest that sexual dimorphism is linked to sexual selection for floral display in males. This theory predicts that for males, having more cones is a reproductive advantage due to increased pollen for wind dispersed species, and a more attractive floral display in zoophilous species. Becoming more highly ramified is suggested to be a strategy in which males can give rise to a greater number of cones, because cones are borne terminally on the ends of branches (Bond and Midgley 1988). Selection for more branching could be associated with subsequent changes in physiology and efficiency of resource acquisition.

It is altogether uncertain whether sexual dimorphism is most influenced by selection on females for more efficient resource acquisition or by selection on male floral display. While both of these hypotheses seem equally intuitive, they are mostly theoretical and there have been no empirical studies showing how male and female physiology may be affected by differing morphologies.

In this study, the physiological implications of sexual dimorphism were investigated in two *Leucadendron* species; the highly dimorphic *L. rubrum* and the non/marginally dimorphic *L. daphnoides* (Both included in Harris and Pannell 2010 study). The study focussed on hydraulic conductance differences between males and females as hydraulic conductance is

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known to limit whole-plant-photosynthetic capacity and maximum assimilation rates (Brodribb and Field 2000; Brodribb 2005, Brodribb et al 2007).

Significantly different hydraulic conductances associated with the different male and female morphologies in highly dimorphic *L. rubrum* would suggest that either differential resource requirements between males and females (due to serotiny), and/or sexual selection on male floral display (with subsequent physiological implications) could have lead to the high degree of sexual dimorphism. Alternatively, no significant hydraulic conductance differences between highly dimorphic *L. rubrum* males and females would suggest that their different morphologies are not closely associated with any physiological costs or benefits. This would further suggest and that differential resource requirements have not been a driving force in sexual dimorphism as males and females would be acquiring resources with comparable efficiency.

If different morphologies are linked to significant physiological differences, *L. daphnoides* males and females are expected to be relatively similar in terms of their hydraulic conductance as they have a low degree of sexual dimorphism. Large hydraulic conductance differences between the morphologically similar *L. daphnoides* males and females would indicate that physiological differences between males and females are not strongly linked to their morphology.

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Methods:

Study Site:

The study site was situated in the Western Cape along the R101, Du Toits Pass (Lat: -33.7107, Long: 19.068085). Co-occurring *Leucadendron rubrum* and *Leucadendron daphnoides* were situated on the slopes extending westward down toward Paarl.

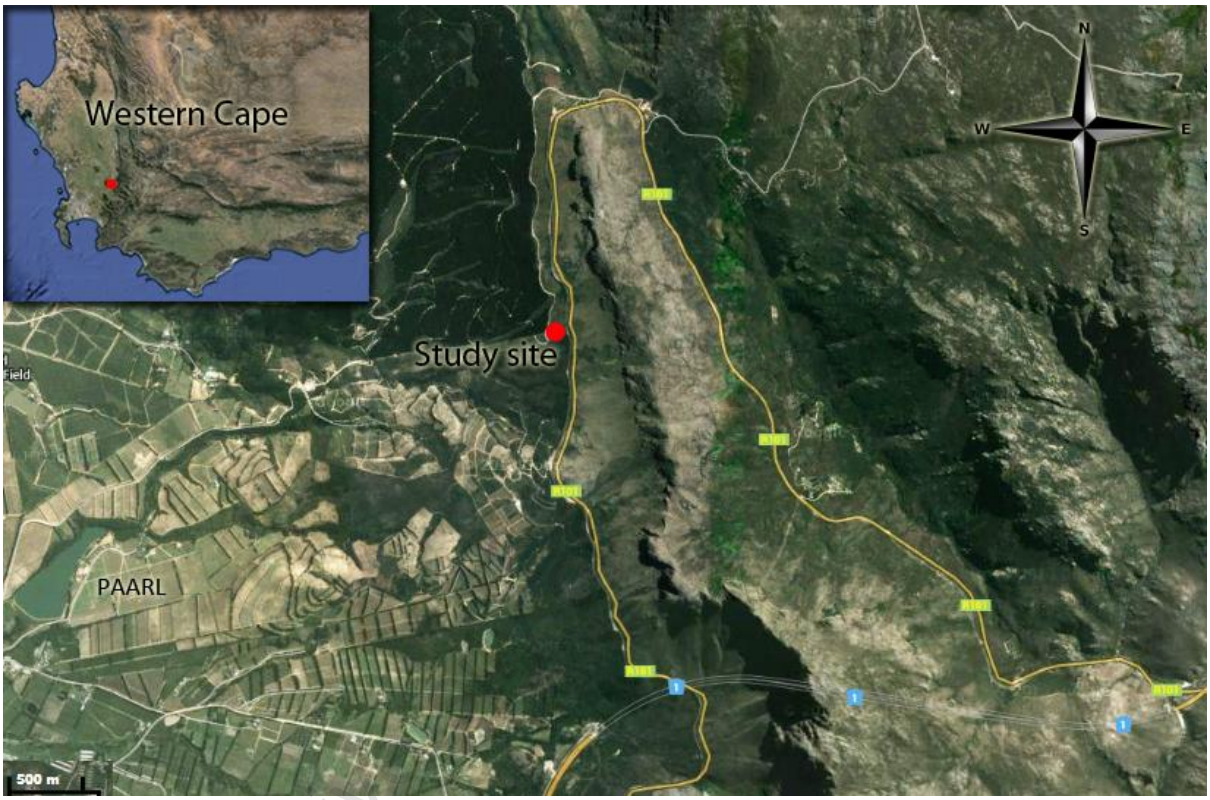

Figure 1: Map of the study area using satellite imagery from Google Earth

In the Field:

Stems including one or more nodes were cut from *Leucadendron rubrum* and *Leucadendron daphnoides* plants using a pair of secateurs. Once cut from the trees, each of these stems was immediately re-cut underwater a few cm from the cut base to prevent air from cavitating the vessels. Each plant was sampled once (single stem removed) to reduce damaging them and an equal sample of 6 male and 6 females was collected for each species.

Once cut, the stems were stored in a bucket of water, each in their own perforated packets to avoid losing and mixing up any leaves. Between processing in the lab, all stems were stored in a 4^0C Dark Storage room. This aided in preventing transpiration and rotting of the stems. Stems left in storage for more than 5 days were thrown out and replaced by new ones.

In the lab: Based on the methods of (Kolb and Sperry 1999)

Each stem was carefully defoliated underwater using sharp, carbon steel blades. Leaves were cut cleanly at the base of the petiole. Care was taken not to break any small branchlets during the defoliation process. Once defoliated, +- 1cm bark around the base of the stem was removed underwater using the same blade. This was to ensure a tight seal in the vacuum chamber. A clean, thin section was then cut from the bottom of the stem to ensure a clean, undisturbed path for the flow of water. The completed defoliated stem was then stored in millipore water until being inserted into the vacuum chamber. Leaves removed from each stem were collected for each stem and their surface area was calculated using a Leaf Area Meter. The number of leaves was also recorded for each stem.

Once inserted into the vacuum chamber, a filtered solution of 0.01mol KCl was then sucked through each defoliated stem at 25, 45 and 65 kPa below atmospheric pressure. Flow rate(kg/s) was recorded using the computer program RSKey and Microsoft Excel. At each pressure, flow rate was recorded a number of times until the coefficient of variation between the different recordings was less than 0.05. This ensures that the KCl solution was flowing through the stem properly and that we were obtaining an accurate flow rate reading.

Hydraulic Conductance of each stem was obtained from the slope of the change in flow between different vacuum pressures.

After calculating Hydraulic Conductance, each stem was flushed with a filtered solution of safranin dye using a simple pressure head, small enough not to induce refilling of any naturally embolised vessels. Stained xylem area for each stem was then measured using photographs of stem cross-sections which were analysed using Image-J.

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Hydraulic Conductance measurements were standardised by dividing by Total Leaf Area or Xylem Area for each stem.

Following the initial analysis, thin cross sections from the base of each stem were cut using sharp carbon steel blades. These were dyed again with safranin dye and analysed under a microscope equipped with a camera. Detailed microscopic images of the stem cross sections on a 1mm graticule microscope slide allowed for the analysis of vessel size between species and sexes. Analysis was done using Image-J.

This data set of 6 males and 6 females for both *Leucadendron* species was combined with another dataset compiled by Adam Roddy (Dec 2012, unpublished), who sampled both *L. rubrum* and *L. daphnoides* individuals from the same study site. In this additional dataset, hydraulic conductance measurements were made following the exact same methodology and using the same vacuum chamber meaning that data were highly comparable. In the additional dataset, xylem area measurements were not made and so particular results associated with xylem area don't include the additional data.

Data analysis and statistics:

All morphological and physiological differences between males and females of each species were analysed using T-tests for independent samples.

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Results

1. Quantifying the degree of sexual dimorphism

1.1 Leaf size

L. rubrum males had a significantly smaller average leaf size than the females, (t(31)=-5.408, p<0.001). Average leaf sizes for the males and females were 0.40cm^2 and 1.07cm^2, respectively. In the male and female *L .daphnoides* individuals, average leaf size was similar; 4.80 cm^2 for the males and 5.10 cm^2 for the females (Fig2).

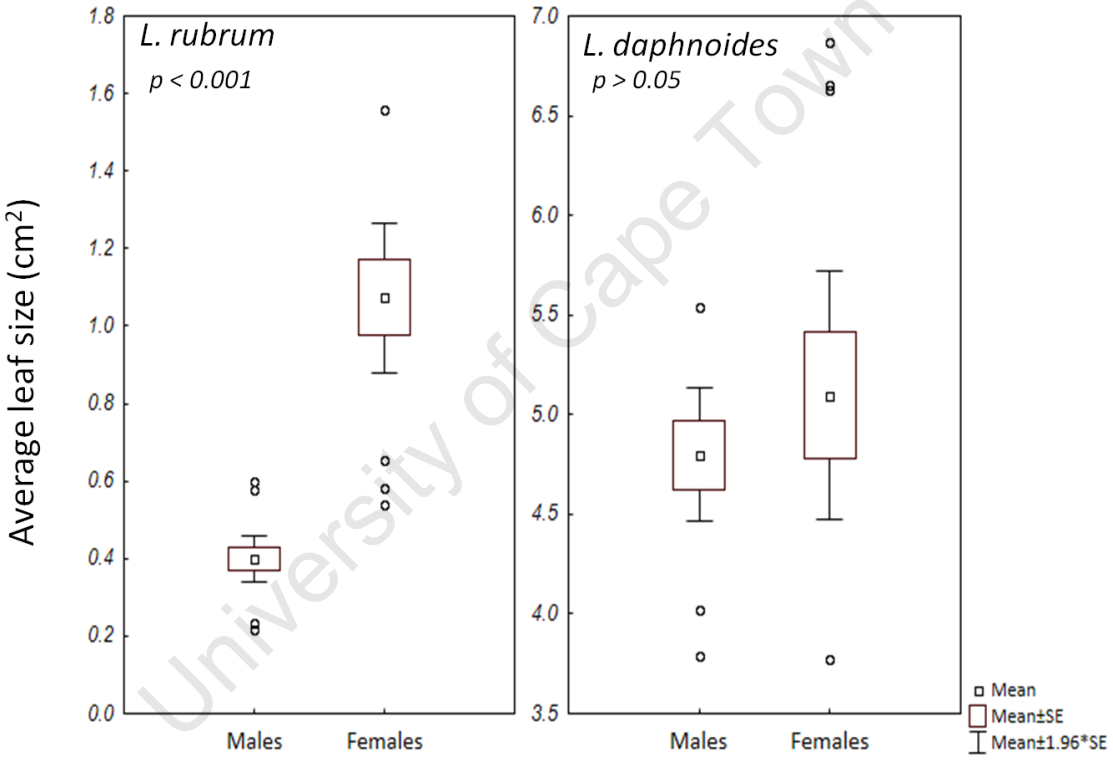

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Figure 2: Average male and female leaf sizes in *L.rubrum* and *L.daphnoides*

1.2 Degree of ramification

The degree of ramification represents the number of branches present relative to the thickness of the stem at the point it was cut in the field. As well as having smaller leaves, *L .rubrum* males were significantly more ramified compared to females, (t(31)=8.50, p<0.001). *L. rubrum* males had many more branches per unit cross-sectional area of stem. As a result of this increased ramification, *L.rubrum* male branches were relatively thinner and more

numerous than the branches of the less ramified females. *L. daphnoides* males and females differ in their degree of ramification to a much lesser extent, ($t(32)=3.16$, $p<0.005$). The variation in ramification was also much lower in *L. daphnoides* compared to *L. rubrum* (Fig3).

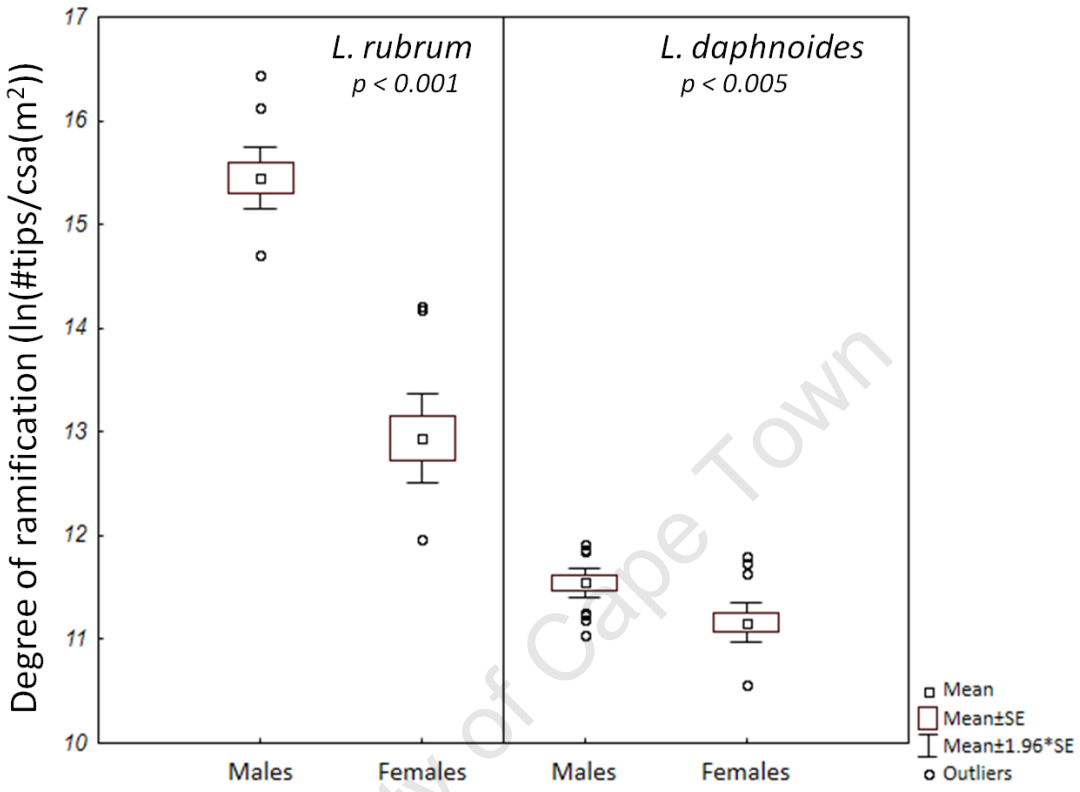

Figure 3: Degree of ramification in males and females of *L. rubrum* and *L. daphnoides*

1.3 Total leaf area: Cross-sectional stem area

Along with their highly branched, small leaf morphology, *L. rubrum* males may have also carried a higher proportion of leaves per stem cross-sectional area compared to females area as they grew larger. While not statistically significant at the α=0.05 level, the mean leaf area: cross sectional stem area of the *L.rubrum* males was 28.77% higher than the females. The difference between the ratios of the male and female *L. daphnoides* was only 7.34% in comparison (Fig4). A scatterplot revealed differences in the slope of the relationship between total leaf area and cross-sectional stem area between males and females of *L. rubrum*. R^2 values suggest that these differences may be biologically significant (Fig5).

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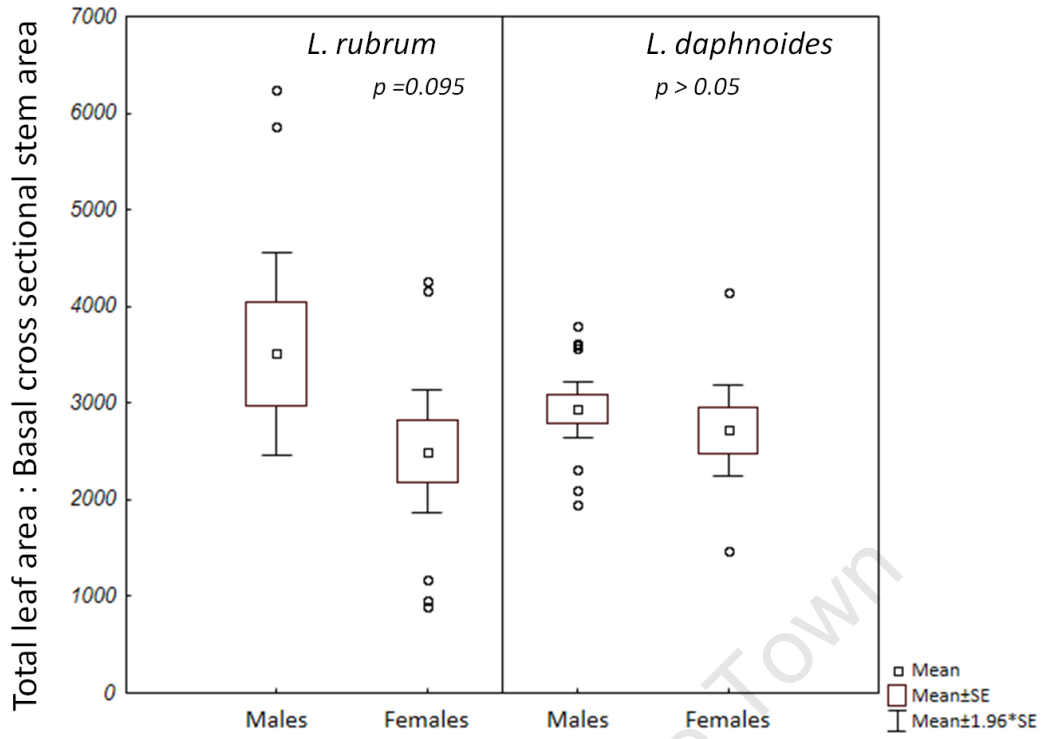

Figure 4: Total leaf area: basal cross-sectional stem area in males and females of *L. rubrum* and *L. daphnoides*

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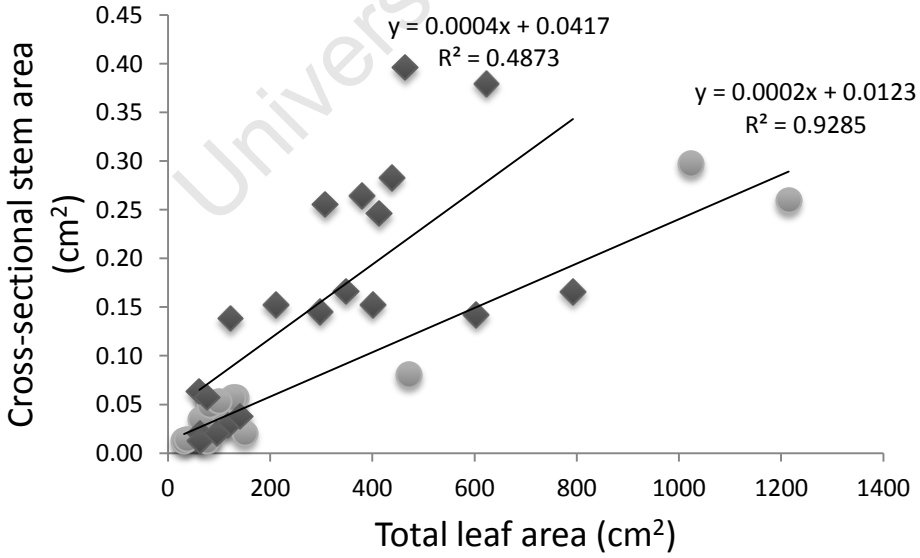

Figure 5: Total leaf area vs cross-sectional stem area in *L. rubrum*. Males = circles. Females = diamonds.

2. Hydraulic Conductance

When compared to the morphologically similar males and females of *L. daphnoides*, it is clear that the *L. rubrum* individuals were highly dimorphic. Differences in their branching and leaf size may be associated with changes in water transport efficiency through the stem.

2.1 Leaf-specific hydraulic conductance

Leaf-specific hydraulic conductance (K_{leaf}) differed significantly between the highly dimorphic *L. rubrum* males and females, ($t(31)=-5.408$, $p<0.001$). Males and females had mean K_{leaf} values of $8.43*10^{-4}$ $kg.s^{-1}.MPa^{-1}.m^{-2}$ and $1.36*10^{-3}$ $kg.s^{-1}.MPa^{-1}.m^{-2}$, respectively (Fig6). No significant difference was found between the K_{leaf} values of the *L. daphnoides* males and females which had mean K_{leaf} values of $1.25*10^{-3}$ $kg.s^{-1}.MPa^{-1}.m^{-2}$ and $1.14*10^{-3}$ $kg.s^{-1}.MPa^{-1}.m^{-2}$, respectively (Fig6).

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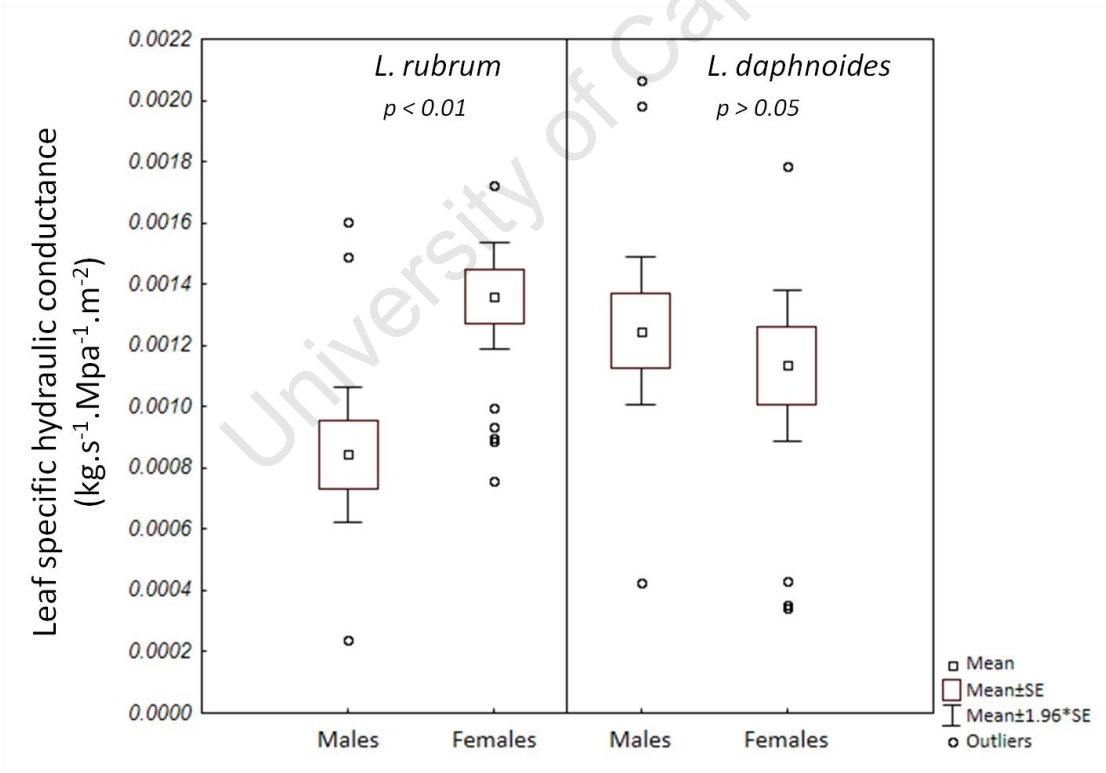

Figure 6: Leaf-specific hydraulic conductance for males and females of *L. rubrum* and *L. daphnoides*

2.2 Xylem-specific hydraulic conductance

Unlike the earlier samples (Roddy 2012, unpublished), stems collected from the site in 2013 had their xylem areas measured. When normalised by xylem area, hydraulic conductance patterns (K_{xylem}) matched the K_{leaf} patterns. *L. rubrum* males had an average K_{xylem} of 3.04 $kg.s^{-1}.MPa^{-1}.m^{-2}$ and females had an average of 4.67 $kg.s^{-1}.MPa^{-1}.m^{-2}$. Although the sample size was small, the differences between *L. rubrum* males and females were significantly different, ($t(10)=-2.85$, $p<0.05$). *L. daphnoides* males had an average K_{xylem} of 6.67 $kg.s^{-1}.MPa^{-1}.m^{-2}$ and females had an average of 8.32 $kg.s^{-1}.MPa^{-1}.m^{-2}$. The difference between *L. daphnoides* males and females was not statistically significant.

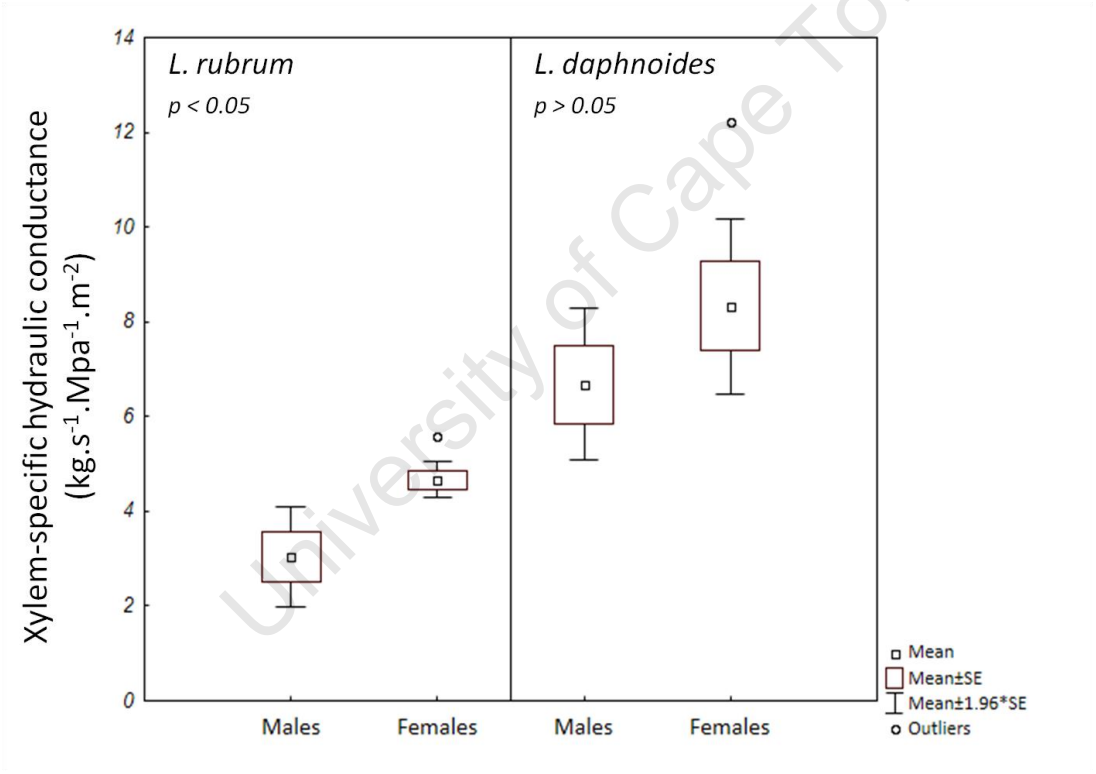

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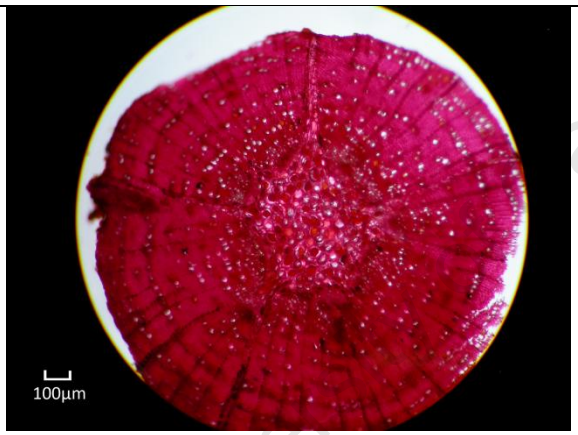
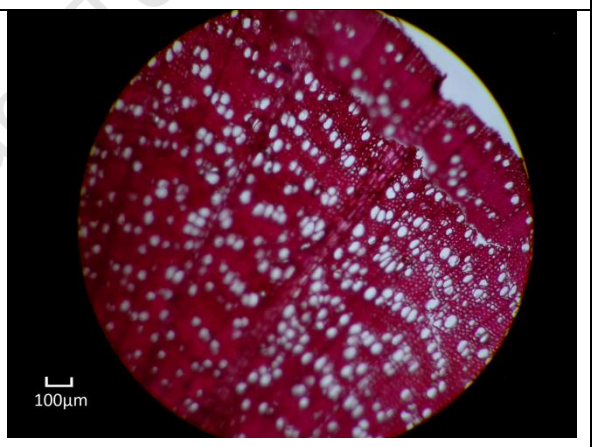
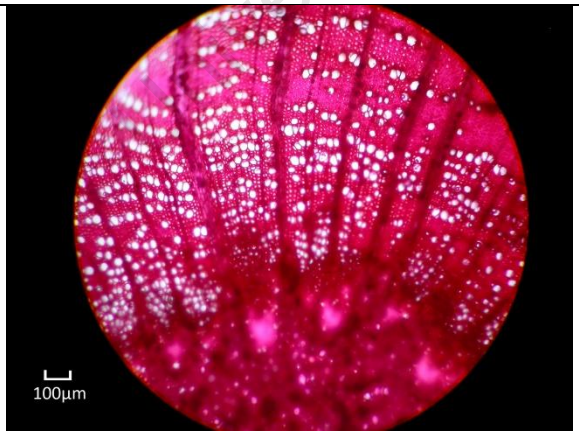
Figure 7: Hydraulic conductance normalised by cross-sectional xylem area for males and females of *L. rubrum* and *L. daphnoides*

3. Xylem anatomy

Hydraulic conductance is likely to be affected by xylem anatomy as differences in vessel size can potentially lead to vastly different flow rates through the stem. Flow rate through a conduit is proportional to the forth power of the conduit diameter. From a visual representation of male and female xylem anatomy it is clear that the male *L. rubrum* xylem tissue had much thinner vessels compared to the females. The *L. daphnoides* xylem tissue appear to be very similar overall (Table 1).

Table 1: Visual comparison of xylem structure between sexes in *L. rubrum* and *L. daphnoides*

	Male	Female
L.rubrum		
L.daphnoides		

Average cross-sectional vessel area of *L. rubrum* males was significantly smaller than the females, (t(9)=-4.29, p<0.01). Males and females had values of $1.83*10^{-4}$ mm^2 and $6.3*10^{-4}$ mm^2 respectively. No significant difference was found between the males and females of the *L. daphnoides* individuals and average values were $4.88*10^{-4}mm^2$ and $4.58*10^{-4}$ $mm^{2,}$ respectively (Fig8).

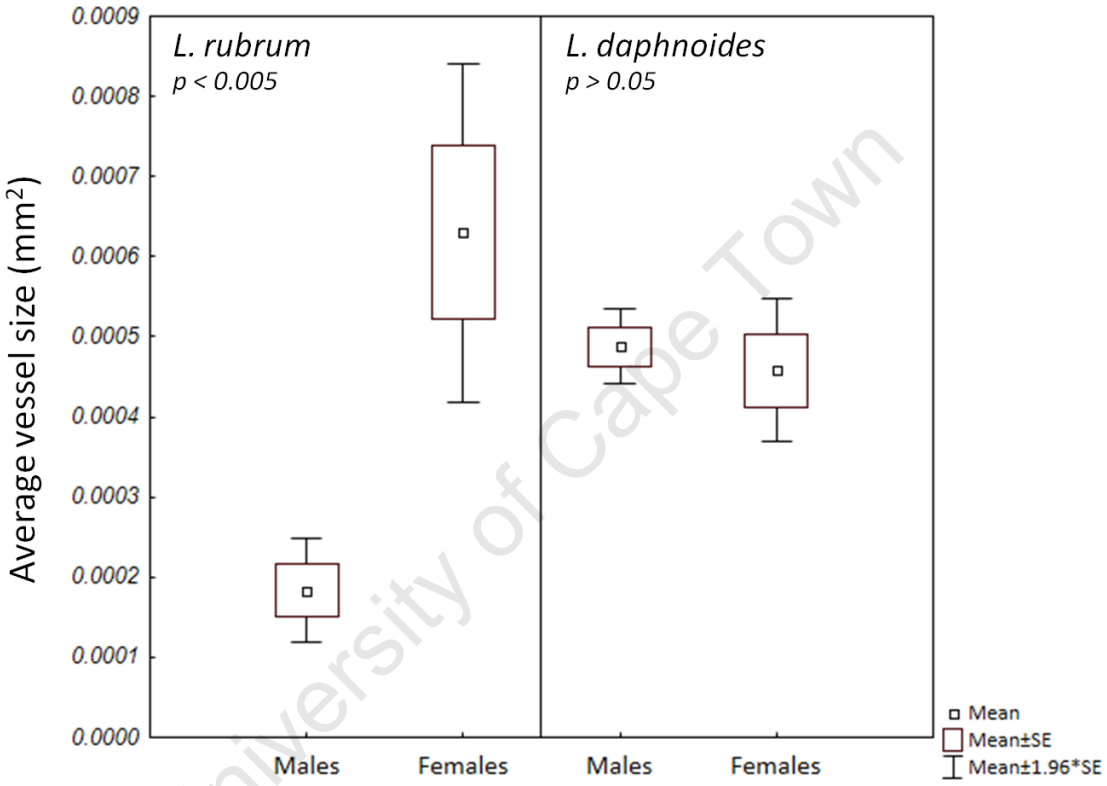


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Figure 8: Average cross-sectional area of vessels in *L. rubrum* and *L. daphnoides* males and females

Discussion

As expected for a highly dimorphic *Leucadendron* species, *L. rubrum* males had a higher degree of ramification and smaller leaves compared to females. Small leaves and a high degree of ramification are closely related due to the allometric relationship between leaf size and stem thickness (Corner 1949). *L. daphnoides* had a much lower degree of sexual dimorphism and males and females were very similar in terms of leaf size and ramification. Therefore, comparing *L. rubrum* and *L. daphnoides* allowed for the identification of physiological changes specifically associated with sexual dimorphism.

Linking morphology and physiology

A significant difference in hydraulic conductance between sexes was present in the highly dimorphic *L. rubrum* individuals but not in the *L. daphnoides* individuals. This suggested that extreme morphological differences between sexes may have given rise to significant physiological differences. Per unit cross-sectional xylem area, *L. rubrum* females had a higher hydraulic conductance than males (Fig7). This is indicative of a more conductive female xylem tissue which appears to have been the result of larger vessels (Fig8 & Table 1). Large vessels transport water at exponentially higher rates compared to small vessels as described by the Hagen Poiseuille equation (1). This equation describes the flow rate of liquid through a conduit. Flow rate is proportional to the forth power of the conduit radius.

(1) $W = \pi \mathbf{D}^4/128\ \eta\ dP/dx$

The *L. rubrum* females also had a significantly higher leaf-specific hydraulic conductance (K_{leaf}) than the males meaning that each unit area of female leaf received a larger supply of water for photosynthesis. Hydraulic supply of water to leaves (K_{leaf}) has been found to be a significant limiting factor influencing the maximum photosynthetic capacity of plants (Broddbribb and Field 2000). Photosynthetic capacity increases with increasing K_{leaf} values allowing for overall higher assimilation rates (Brodribb and Field 2000; Brodribb 2005, Brodribb et al 2007). This can be explained through the stomatal regulation of leaf gas exchange. The uptake of CO2 and the transpiration of water vapour both take place through stomata which can be opened or closed to balance evaporative demand with water supply (Brodribb 2005). It follows that if water supply is limited through low xylem conductivity,

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down regulation of stomatal conductance will limit the uptake of C02 and overall maximum assimilation will be constrained. Overall, the *L. rubrum* female morphology allowed for a highly conductive xylem tissue and leaves that are well supplied with water for transpiration and photosynthesis.

Evidence also suggests that *L. rubrum* males may have had more leaf area per stem to supply with water than females (Fig4 &5). This is also likely to have contributed to the lower K_{leaf} values of *L. rubrum* males as their limited water supply would have to be split between a higher total leaf area. The cost of producing and maintaining extra leaf area, supplied with relatively less water, means that the males were less efficient in terms of their growth strategy. Physiological differences appeared to be specifically linked to a high degree of sexual dimorphism as the morphologically similar *L. daphnoides* males and females were physiologically similar.

Significance of hydraulic differences in an evolutionary context

The evidence from this study does not refute the hypothesis put forward by Harris and Pannell 2010 who suggested that females of highly serotinous species (such as *L. rubrum*) incur extra resource costs due to the fact that their cones must transpire to be kept alive over a number of years between fires (Harris and Pannell 2010). Due to this increase in resource requirements, a strategy for more efficient resource acquisition may have been selected for. Although the evidence suggests that females are indeed more efficient than the males, this may have been the ancestral condition and may not have been selected for in order to acquire extra resources. It is equally likely that selection for more branching in males came with hydraulic costs, lowering the efficiency of male hydraulic transport from the more efficient ancestral condition. Alternatively, an ancestral morphology somewhere in between the two current male and female extremes could have diverged due to different selective pressures on both the males and females.

As suggested by Bond and Midgley 1988, selection for a higher degree of ramification in *L. rubrum* males would be a strategy to increase the number of terminally borne inflorescences which would in turn increase pollination success (Bond and Midgley 1988). If this is the case, lower hydraulic conductivity and thus lower resource use efficiency in males could be the subsequent cost associated with selection for more branches. For example, in

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Leucadendron xanthoconus, extravagant floral display in males lead to a higher probability of death due to higher carbon costs and reduced carbon uptake associated with their morphology (Bond and Maze 1999). Due to the high degree of ramification in *L. rubrum* males, their branches were relatively thinner than females. With thin branches, males may be constrained to having small vessel sizes as xylem tissue occupies a limited space in the stem. Theoretically this space could either host numerous small, or few large vessels. Having many small vessels is a much safer option for the males as the risk of a high proportion of vessels becoming embolised or cavitated is much lower. If a high enough proportion of vessels in the xylem tissue become non-functional, the plant faces a serious loss of photosynthetic capacity or even death (Hubbard et al 2001; McDowell et al 2008). Large vessels are also more prone to cavitation than small ones (Brodribb et al 2012) and having few overall would increase the risk of a catastrophic loss in conductivity. It has further been shown that nodal resistance could play a role in reducing overall hydraulic conductivity (Slingsby 2004). A higher frequency of nodes associated with increased ramification could therefore also play a role in reducing the hydraulic conductance in *L. rubrum* males.

Thus far the possibility of selection for different leaf sizes has not been addressed. While it is more likely that leaf size is a secondary result of their allometric relationship with branch thickness (Corner 1949), the possibility remains that selection for larger or smaller leaves could have driven other morphological differences. The relationships between leaf size and photosynthesis are complex and were not directly addressed in this study, however, the ecophysiological implications of leaf size have been investigated in 15 species of Cape Proteaceae (Yates et al 2010). It was suggested that small leaf size may be associated with higher rates of transpiration when evaporative demand is low (eg: in winter) while allowing for sensible heat loss in summer, lowering the need for transpirational cooling and thus lowering the amount of water stress (Yates et al 2010). It is unclear whether the advantages associated with small leaves would have been selected for in males or whether leaf size is the subsequent result of its allometric with stem thickness (Corner 1949). If small leaf size was indeed a trait that was directly selected for in males, the question remains as to why females did not experience the same selective pressure, unless higher resource requirements acted as a counter to such selection. To complicate matters further, differential transpiration in males and females due to leaf size, could interact with hydraulic

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supply and affect whole plant photosynthesis. Higher hydraulic conductance in females could possibly be associated with additional transpirational cooling requirements due to larger leaves. These issues were not directly dealt with in this study and further research is required to untangle these complex relationships.

Future improvements of the study

Future improvements in studies such as this one could be achieved through the inclusion of a wider range of *Leucadendron* species, with varying degrees of sexual dimorphism. It would be interesting to see if the amount of difference in hydraulic conductivity between dimorphic males and females is positively correlated to the degree of sexual dimorphism. If the hydraulic conductance values of a wide range of *Leucadendron* species is known (including non-dimorphic spp), deducing whether the female or male morphology in any dimorphic species is more derived would be possible through comparison to the norm. This would shed light on whether selection has acted primarily on males for floral display, females for resource acquisition or a combination of both. Further research on *Leucadendron* plant hydraulics also needs to include the effects of leaf size and stomatal density. This will allow for a more complete understanding of physiological differences and their effects on whole plant photosynthesis, assimilation and resource use.

Significance of study

This study provides the first empirical evidence showing that the morphological differences in males and females of highly dimorphic species are associated with physiological costs and benefits. Highly branched males with small leaves appear to have a less conductive xylem tissue which is less efficient at supplying leaves with water. This has previously been shown to result in an overall lower photosynthetic capacity. The evidence from this study can be used to support the hypothesis that highly serotinous females have evolved morphologically to be more efficient at resource acquisition (Harris and Pannell 2010) but can also support the hypothesis that selection for more attractive floral display in males lead to a high degree of ramification with subsequent physiological costs (Bond and Midgley 1988). Further research is thus required to compare the strength of these two hypotheses.

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University of Cape Town

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